Figure 1: Body weight of male and female castrated rats given HDBB by gavage for 28 days.

Table 1: Blood biochemical findings in male and female castrated rats given HDBB by gavage for 28 days.

Dose (mg/kg/day)	0	0.5	2.5	12.5
Male				
No. of animals	10	10	10	10
Total protein (g/dL)	6.19 ± 0.32	6.44 ± 0.23	6.45 ± 0.40	6.26 ± 0.31
Albumin (g/dL)	4.43 ± 0.18	4.90 ± 0.17**	4.99 ± 0.25**	5.03 ± 0.18**
AST (IU/L)	61.0 ± 6.2	54.4 ± 3.5	63.6 ± 8.0	91.4 ± 24.0**
ALT (IU/L)	40.2 ± 8.9	37.9 ± 4.2	46.2 ± 8.6	55.5 ± 7.2**
ALP (IU/L)	868 ± 200	995 ± 267	989 ± 344	1552 ± 538**
LDH (IU/L)	112 ± 28	129 ± 18	173 ± 30*	403 ± 189**
Glucose (mg/dL)	176 ± 12	199 ± 13**	176 ± 7	196 ± 22*
BUN (mg/dL)	15.8 ± 2.0	15.3 ± 2.1	16.0 ± 1.8	19.7 ± 1.6**
Creatinine (mg/dL)	0.208 ± 0.020	0.174 ± 0.022**	0.176 ± 0.027**	0.175 ± 0.016**
Na (mEq/L)	145 ± 1	145 ± 1	145 ± 1	142 ± 1**
Cl (mEq/L)	107 ± 1	106 ± 2	106 ± 2	104 ± 2**
Female				
No. of animals	10	10	10	9[a]
Total protein (g/dL)	5.81 ± 0.21	6.17 ± 0.26**	6.15 ± 0.18*	6.41 ± 0.34**
Albumin (g/dL)	4.19 ± 0.12	4.39 ± 0.22	4.55 ± 0.19**	5.14 ± 0.32**
AST (IU/L)	54.8 ± 3.5	62.4 ± 5.1*	57.4 ± 6.2	58.4 ± 10.0
ALT (IU/L)	39.1 ± 4.6	43.2 ± 7.8	39.5 ± 5.9	45.8 ± 8.7
ALP (IU/L)	727 ± 164	742 ± 122	703 ± 199	1026 ± 217**
LDH (IU/L)	138 ± 44	254 ± 27**	209 ± 44*	235 ± 116*
Glucose (mg/dL)	202 ± 25	181 ± 13	182 ± 10	216 ± 16
BUN (mg/dL)	20.0 ± 1.6	20.2 ± 2.1	18.2 ± 2.6	23.2 ± 2.2**
Creatinine (mg/dL)	0.230 ± 0.022	0.229 ± 0.025	0.196 ± 0.022*	0.208 ± 0.030
Na (mEq/L)	142 ± 1	143 ± 1	144 ± 1**	141 ± 1
Cl (mEq/L)	104 ± 1	105 ± 2	106 ± 1**	102 ± 2*

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]One female was excluded because left ovary remnants were found at autopsy.

The levels of LDH at 2.5 mg/kg/day and above in males and at 0.5 mg/kg/day and above in females, ALP at 12.5 mg/kg/day in both sexes, and AST and ALT at 12.5 mg/kg/day in males were also significantly increased. In addition, significant decreases in the levels of creatinine at 0.5 mg/kg/day and above, of sodium at 12.5 mg/kg/day in males, and of chloride at 12.5 mg/kg/day in both sexes were detected.

At necropsy, no gross abnormality was found at any dose. Absolute and relative liver weight was significantly increased at 0.5 mg/kg/day and above in males and at 12.5 mg/kg/day in females (Table 2). No significant change was found in the absolute and relative heart weight.

Histopathological findings in the liver are summarized in Table 3. Diffuse hypertrophy of hepatocytes were observed at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. The cytoplasm of the hepatocytes was slightly eosinophilic. At these doses, anisokaryosis, nucleolar enlargement, and decreased glycogen in hepatocytes were also found. In addition, focal coagulative necrosis at 12.5 mg/kg/day in males and at 2.5 mg/kg/day and above in females, and increased mitosis of hepatocytes at 2.5 mg/kg/day and above and mononuclear cell infiltration at 12.5 mg/kg/day in males, were detected. No substance-related histopathological findings were detected in the heart.

DISCUSSION

The current study was designed to investigate the role of sex steroids in the mediation of gender-related differences in HDBB toxicity. The dosage of HDBB used in the present study was sufficiently high to be expected to induce

Table 2: Organ weight of the heart and liver in male and female castrated rats given HDBB by gavage for 28 days.

Dose (mg/kg/day)	0	0.5	2.5	12.5
Male				
No. of animals	10	10	10	10
Heart (g)	1.30 ± 0.07	1.25 ± 0.09	1.35 ± 0.12	1.37 ± 0.12
	(0.352 ± 0.022)[a]	(0.331 ± 0.028)	(0.362 ± 0.020)	(0.373 ± 0.030)
Liver (g)	15.5 ± 1.5	18.2 ± 2.7*	21.6 ± 3.0**	26.9 ± 1.9**
	(4.18 ± 0.27)	(4.78 ± 0.47*)	(5.76 ± 0.61**)	(7.32 ± 0.40**)
Female				
No. of animals	10	10	10	9[b]
Heart (g)	1.14 ± 0.07	1.11 ± 0.09	1.15 ± 0.10	1.25 ± 0.14
	(0.342 ± 0.027)	(0.322 ± 0.027)	(0.329 ± 0.024)	(0.352 ± 0.035)
Liver (g)	14.5 ± 1.9	14.8 ± 1.4	16.2 ± 2.5	27.0 ± 3.3**
	(4.33 ± 0.34)	(4.28 ± 0.19)	(4.63 ± 0.32)	(7.63 ± 0.87**)

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Relative organ weight (g/100 g body weight).
[b]One female was excluded because left ovary remnants were found at autopsy.

Table 3: Histopathological findings in the liver of male and female castrated rats given HDBB by gavage for 28 days.

	Grade	Dose (mg/kg/day)			
		0	0.5	2.5	12.5
Male					
No. of animals		10	10	10	10
Anisokaryosis of hepatocytes	±	0	1	8	3
	+	0	0	0	7
Nucleolar enlargement in hepatocytes	±	0	1	10	5
	+	0	0	0	5
Increased mitosis of hepatocytes	±	0	0	1	4
Hypertrophy of hepatocytes	±	0	4	10	10
Decreased glycogen in hepatocytes	±	0	1	6	8
	+	0	0	0	2
Focal necrosis	±	0	0	0	3
Mononuclear cell infiltration	±	1	0	0	5
Female					
No. of animals		10	10	10	9[a]
Anisokaryosis of hepatocytes	±	0	0	5	8
Nucleolar enlargement in hepatocytes	±	0	0	5	9
Hypertrophy of hepatocytes	±	0	0	2	9
Decreased glycogen in hepatocytes	±	0	0	2	8
Focal necrosis	±	0	0	3	2
Mononuclear cell infiltration	±	1	1	1	0

Values represent the number of animals with the findings.
± = very slight; + = slight.
[a]One female was excluded because left ovary remnants were found at autopsy.

toxicological effects on the liver, based on the results of the previous 28-day and 52-week repeated dose toxicity study using intact rats (Hirata-Koizumi et al., 2007; 2008). As expected, absolute and relative liver weight increased at 0.5 mg/kg/day and above in males and at 12.5 mg/kg/day in females, and histopathological changes in the liver, including anisokaryosis, nucleolar enlargement, increased mitosis, hypertrophy and decreased glycogen in hepatocytes, focal necrosis, and/or mononuclear cell infiltration, were observed at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. Blood biochemical changes, such as increases in the level of total protein, albumin, AST, ALT, ALP, and/or LDH, were also found at all doses in both sexes. Although these changes in blood biochemical parameters were mostly slight and lacked dose dependence in some cases, simultaneous increase in hepatic enzymes (AST, ALT, ALP, and LDH) at 12.5 mg/kg/day in males is considered to be related to hepatic damage caused by HDBB.

A previous 28-day study using intact rats showed the cardiac toxicity of HDBB; degeneration and hypertrophy of the myocardium or cell infiltration were found at 2.5 mg/kg/day and above in males and at 12.5 mg/kg/day and above in females (Hirata-Koizumi et al., 2007). In the present study, using castrated rats, however, histopathological changes in the heart were not

detected even at the highest dose of 12.5 mg/kg/day. Considering that histopathological effects on the heart were also not found at the highest dose of 2.5 mg/kg/day in males and 12.5 mg/kg/day in females in the previous 52-week study using intact rats (Hirata-Koizumi et al., 2008), the present results would not necessarily mean that castration caused a change in the cardiac effect of HDBB. Although the cause of the difference in the cardiac toxicity of HDBB in our studies is not clear, further study is required to investigate the toxicological effects of HDBB on the heart in more detail, including the effect on cardiac function (e.g., electrocardiographic parameters, blood pressure, etc.).

In the previous 28-day study, male and female intact rats were given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day (Hirata-Koizumi et al., in press). Histopathological findings similar to those observed in the present study were detected in the liver at all doses in males and at 12.5 mg/kg/day and above in females. The changes were accompanied with an increase in the absolute and/or relative liver weight. Serum levels of hepatic enzymes increased at 12.5 mg/kg/day and above in males and slightly at 62.5 mg/kg/day in females. When comparing the sensitive endpoint for hepatotoxicity of HDBB, histopathological changes in the liver, between sexes, the changes detected at 0.5 mg/kg/day in male rats were comparable in severity and incidence to those at 12.5 mg/kg/day in females. Thus, it was considered that male rats showed a nearly 25 times higher susceptibility to the hepatotoxicity of HDBB than females. In the present study, using castrated rats, histopathological findings in the liver were detected in males but not in females at the lowest dose of 0.5 mg/kg/day. The hepatic changes at 0.5 mg/kg/day in males were slightly milder than those at 2.5 mg/kg/day in females, showing that the difference in the susceptibility of male and female castrated rats was less than five times. Thus, castration markedly reduced gender-related differences in the hepatotoxicity of HDBB. As shown in Figure 2, a comparison of the rate of changes in the relative liver weight provided a more clear description of a nearly 25 times difference in the susceptibility of male and female intact rats to HDBB hepatotoxicity and the marked reduction by castration.

When comparing the histopathological findings of the liver from the previous 28-day study using intact rats and the present study using castrated rats, those in males were approximately equivalent at the same dose. On the other hand, for females, hepatic changes were observed at 12.5 mg/kg/day and above in intact rats, but clear changes in the histopathology of the liver were detected in castrated rats at a lower dose of 2.5 mg/kg/day. Therefore, castration of female rats enhanced the adverse effects of HDBB on the liver, suggesting suppressive effects of estrogen on HDBB hepatotoxicity in rats. Comparison of the relative liver weight change (Fig. 2) showed decreased male susceptibility as well as increased female susceptibility by castration. Androgen might have an enhancing effect on the hepatotoxicity of HDBB.
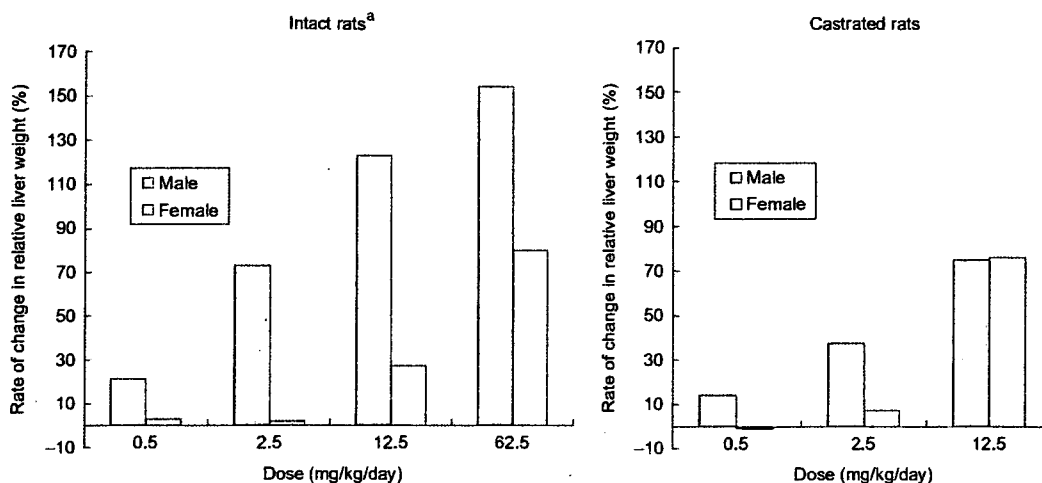
Figure 2: Comparison of change in relative liver weight of male and female intact and castrated rats given HDBB by gavage.

[a]The result of the previous 28-day repeated dose toxicity study, in which male and female intact rats were given HDBB once-daily at 0 (vehicle control), 0.5, 2.5, 12.5, and 62.5 mg/kg/day by gavage (Hirata-Koizumi et al., 2007)

The current study showed that the gender-related difference in susceptibility to HDBB hepatotoxicity was reduced, but not abolished, by castration. Sexual differences found in the present study were considered to be due to exposure to sexual hormones before four weeks of age, when castration was conducted. In female rats, serum estradiol concentration during the first three weeks after birth is as high as or higher than the level during the proestrus stage in young adults (Döhler and Wuttke, 1975); however, because serum estradiol concentration is similarly high during this preweaning period in male rats, it is unlikely that exposure to estradiol during this period contributes to the sexual difference in susceptibility of rats to the toxicity of HDBB. On the other hand, serum androgen levels before four weeks of age are much higher in male than female rats (Döhler and Wuttke, 1975). Ketelslegers et al. (1978) reported that plasma testosterone level in male rats was as high as 50 ng/100 mL two days after birth and it remained at the same level until day 8. The progressive decline occurred from days 8–24, and the testosterone level remained low, at the limit of detection of the assay (18 ng/100 mL), until day 30. There is a possibility that neonatal exposure to testosterone plays some role in the different susceptibility of male and female rats to the toxicity of HDBB. In fact, neonatal exposure to androgen irreversibly programs brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent metabolism (Gustafsson et al., 1981). We are currently in the process of performing a repeated dose toxicity study of HDBB using preweaning rats to clarify when gender-related differences in susceptibility to the toxicity of HDBB develop.

As in the case of HDBB, the male-predominant induction of toxicity in rats has been reported for many other substances, such as adenine (Ogirima et al.,

2006), acetaminophen (Raheja et al., 1983), dapsone (Coleman et al., 1990), fluoranthene (Knuckles et al., 2004), 3-nitropropionic acid (Nishino et al., 1998), and mercuric chloride (Muraoka and Itoh, 1980). Various causes of such gender-related differences are indicated mainly for toxicokinetic determinants. It is well known that hepatic metabolism differs between the sexes, with male rats generally having higher activity than females (Gad, 2006). Furthermore, gender differences in membrane transport in various organs, including the kidneys, liver, intestine, and brain, have emerged relatively recently (Morris et al., 2003). In the case of HDBB, male rats consistently showed greater susceptibility to various effects of HDBB (e.g., on the liver, blood, etc.) in the previous 28-day and 52-week studies (Hirata-Koizumi et al., 2007; 2008); therefore, such differences in metabolism or transport between the sexes might increase the blood concentration of causative substances (i.e., HDBB or its metabolites) in males.

For gender-related variations in toxicokinetic determinants, many mechanistic studies on the metabolic enzyme cytochrome P450 have been reported (Waxman and Chang, 2005). In rats, a subset of P450s is expressed in a sex-dependent fashion and is subject to endocrine control. Whereas testosterone has a major positive regulatory influence on male-specific P450 forms, estrogen plays a somewhat lesser role in the expression of the female-specific/predominant liver P450 enzymes. If the male-specific/predominant metabolic enzymes have an intimate involvement in the toxic activation of HDBB, our results, showing the higher susceptibility of male rats to HDBB toxicity than females and decreased susceptibility by castration of male rats, could be explained. Interestingly, it was reported that estradiol suppressed the expression of male-specific/predominant P450 enzymes (Waxman and Chang, 2005). This is consistent with our results that female susceptibility to the hepatotoxicity of HDBB was increased by castration, given that the male-specific/predominant P450 enzymes activate HDBB. Since the expression of female-specific/predominant P450 enzymes is reduced by testosterone treatment as well as by castration of females (Waxman and Chang, 2005), there is also the possibility that these enzymes might be involved in the detoxication of HDBB. In order to clarify the cause of the sexual differences in susceptibility of rats to the toxicity of HDBB, we are planning a toxicokinetic study of HDBB, which would include the identification of metabolites and the related metabolic enzyme as well as measurement of the blood concentration of HDBB both after a single and repeated administration of HDBB to rats.

CONCLUSIONS

The current results showed that an oral administration of HDBB to castrated rats for 28 days caused hepatotoxicity at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. Castration markedly reduced gender-related differences in the toxicity of HDBB in male and female rats.
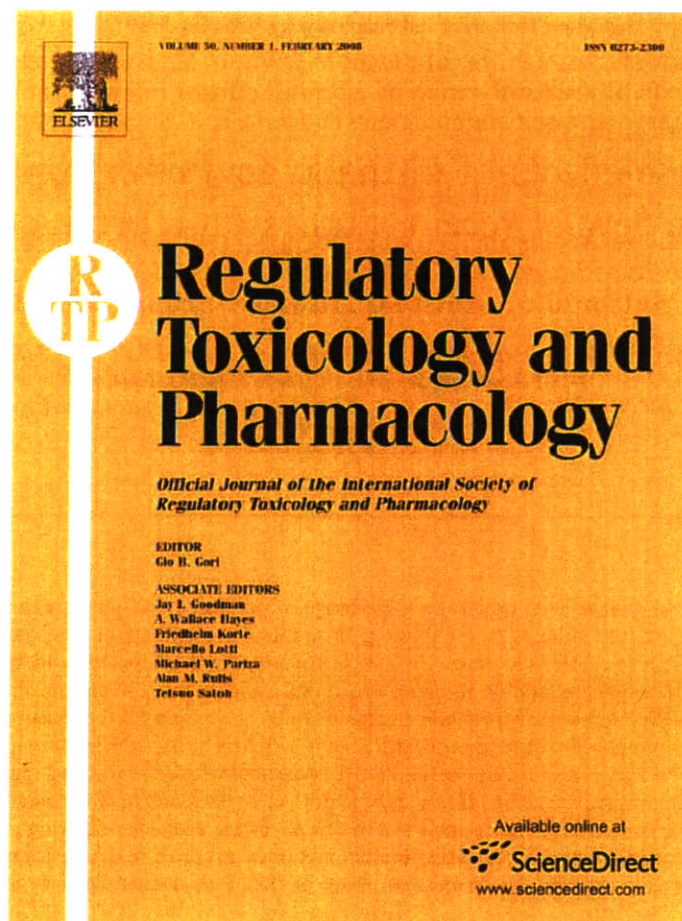
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Potential adverse effects of phthalic acid esters on human health: A review of recent studies on reproduction

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Abstract

Various phthalic acid esters (PAEs) have been used for a wide range of products. PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. This paper focuses on PAE exposure and health effects in human populations and summarizes recent studies. The exposure data in human populations indicate that the current methodology of estimation of PAE exposure is inconsistent. It is therefore important to obtain improved data on human PAE exposure and better understanding of the toxicokinetics of PAEs in each subpopulation. Studies on health effects of PAEs in humans have remained controversial due to limitations of the study designs. Some of findings in human populations are consistent with animal data suggesting that PAEs and their metabolites produce toxic effects in the reproductive system. However, it is not yet possible to conclude whether phthalate exposure is harmful for human reproduction. Studies in human populations reviewed in this paper are useful for showing the strength of the association. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, because it is difficult to find alternative methods to examine the direct toxic effects of chemicals, animal studies remain necessary for risk assessment of chemicals including PAEs.
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Keywords: Phthalic acid ester; Human health; Reproduction; Development

1. Introduction

Various phthalic acid esters (PAEs) have been used for a wide range of products, and the largest use of these esters is in plasticizers for polyvinyl chloride (PVC) products (Autian, 1973). When used as plasticizers, PAEs are not irreversibly bound to the polymer matrix; therefore, they can migrate from the plastic to the external environment under certain conditions. PAEs are ubiquitous environmental pollutants because of their widespread manufacture, use, and disposal as well as their high concentration in and ability to migrate from plastics (Marx, 1972; Mayer et al., 1972). Humans are exposed to PAEs from food con-

taminated during growth, processing, and packaging or from storage and indoor air. Di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) were particularly found in fatty foods including dairy products (Kavlock et al., 2002a,b,c). Women have been exposed to DEHP, DBP, and diethyl phthalate (DEP) in cosmetics on a daily basis (Koo and Lee, 2004).

Some PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. The major toxicities are known to be testicular effects (Zhang et al., 2004), embryolethality (Ema et al., 1994, 1997a; Tyl et al., 1988), malformations such as cleft palate and fusion of the sternebrae, and adverse effects on sexual differentiation (Ema et al., 1997b, 1998; Gray et al., 2000). There are considerable homologies among different

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mammalian species for androgen activities during sex differentiation (Gray et al., 1994). Chemicals that adversely affect human sex differentiation (Schardein, 2000) also produce predictable alterations of this process in rodents (Gray et al., 1994). The anti-androgenic effects of some PAEs were observed in a Hershberger assay in castrated male rats (Stroheker et al., 2005; Lee and Koo, 2007) or in an AR reporter gene assay (Satoh et al., 2004). These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. Available data on primates are currently limited but show significant differences from rodents regarding the reproductive effects of PAEs (Kurata et al., 1998; Pugh et al., 2000; Tomonari et al., 2006), indicating the possibility of species-related differences.

The lower sensitivity of primates is thought to arise from differences between rodents and primates in the absorption, distribution, metabolism, and excretion (ADME) of PAEs. Monoester metabolites of PAEs such as mono-2-ethylhexyl phthalate (MEHP) and mono-butyl phthalate (MBP) have been reported to be the active metabolites responsible for adverse effects (Elcombe and Mitchell, 1986; Ema and Miyawaki, 2001; Tomita et al., 1986). DEHP is hydrolyzed to MEHP by the catalytic action of lipase (Ito et al., 2005). Lipase activities in the liver, small intestine, and kidneys are higher in rodents than in primates (Ito et al., 2005). The maximum concentrations of MEHP in the blood of marmosets were up to 7.5 times lower than in rats (Kessler et al., 2004). In rats, MEHP is oxidized to other secondary metabolites, and both MEHP and secondary metabolites are found in the blood and amniotic fluid primarily in their free form (Kurata et al., 2005; Calafat et al., 2006). Urinary MEHP was mostly found as a glucuronide conjugate in rats (Calafat et al., 2006). On the other hand, in humans and primates, MEHP is present in blood and urine primarily as glucuronide conjugates, which enhance urinary excretion and reduce the biological activity of the active metabolites (Ito et al., 2005; Kurata et al., 2005; Silva et al., 2003), but DEHP metabolites with a carboxylated ester side-chain were found as both conjugates and free forms in human urine (Silva et al., 2006a). Plasma radioactivity measurements of DEHP in rats and marmosets revealed that radioactivity in rat testis was about 20-fold higher than that in marmosets. About 60% of the dose was excreted in urine in rats primarily as unconjugated MEHP-metabolites. For marmosets, the majority of the dose was excreted in the feces (Kurata et al., 1998).

The potential of PAEs to produce adverse effects in humans has been the subject of considerable discussion. Many toxicity studies have been conducted in laboratory animals, especially in rats, and review papers are available based on these animal data (Corton and Lapinskas, 2005; Ema, 2002; Foster, 2006); however, studies in human populations have not been adequate to assess the toxic potential on human health. Lately, several review papers were published regarding PAE exposure in human populations (Koch et al., 2006; Latini, 2005; Schettler, 2006). These review studies are worthwhile for knowing exposure levels and routes of PAE exposure in human populations; however, review works regarding the relationships between PAE exposure and human health are not adequate. In the late 20th century, only a few papers have reported a relationship between environmental PAE exposures and human health (Aldyreva et al., 1975; Fredricsson et al., 1993; Murature et al., 1987). Studies in human populations have been receiving much attention for the last 2 or 3 years, and the number of studies in human populations has increased. Some recent studies have suggested possible associations between environmental exposure to PAEs and adverse effects on human reproductive health. It will be useful to review them to determine whether there is concordance between animal models and human populations in order to develop hypotheses for future studies. This paper focuses on the PAE exposure and health effects in human populations and summarizes recent human studies published up to 2006.

2. Exposure to PAEs

Many studies have suggested that PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. Although the most of these animals were exposed to PAEs at relatively high level to exam toxicological effects, some studies showed that relatively low doses of PAEs caused toxic effects (Arcadi et al., 1998; Lee et al., 2004; Poon et al., 1997). Thus, there is a question of whether humans are exposed to PAEs at a severe enough level to generate human health effects. Several studies have been conducted to estimate the exposure level of PAEs in humans.

2.1. Estimate of PAE exposure in human populations

Levels of human exposure to PAEs were estimated from the urinary metabolite of PAEs. Table 1 shows the urinary PAE metabolite in US populations. A pilot study was conducted for measurement of levels of seven urinary phthalate metabolites, MEHP, MBP, mono-benzyl phthalate

Table 1
Total urinary phthalate monoester concentrations (in µg/g of creatinine)

Metabolites	Diester	Measurement in 289 individuals (Blount et al., 2000)		Measurement in 2541 individuals (Silva et al., 2004a)	
		Geometric mean	95th percentile	Geometric mean	95th percentile
MEP	DEP	345	2610	163	1950
MBP	DBP/BBP	36.9	162	22.4	97.5
MBzP	BBP	20.2	91.9	14.0	77.4
McHP	DcHP	0.3	1.0	<LOD	3.00
MEHP	DEHP	3.0	15.2	3.12	18.5
MOP	DOP	0.5	2.1	<LOD	3.51
MINP	DINP	1.3	6.8	<LOD	4.29

LOD, limit of detection.

(MBzP), mono-cyclohexyl phthalate (McHP), mono-ethyl phthalate (MEP), mono-isononyl phthalate (MINP) and mono-n-octyl·phthalate (MOP), in 289 US adults (Blount et al., 2000). A subsequent study involving a group of 2541 individuals from participants of the National Health and Nutrition Examination Survey (NHANES) aged ⩾ 6 years in US provided similar findings to the previous study although urinary levels for MEP, MBP and MBzP were lower than the previously reported values (Silva et al., 2004a).

These urinary metabolite levels were used to calculate the ambient exposure levels for five PAEs, BBP, DBP, DEHP, di-n-octyl phthalate (DOP) and di-isononyl phthalate (DINP), in human populations (David et al., 2001; Kohn et al., 2000). The estimation of daily intake of phthalates was calculated by applying the following equation according to David et al. (2001):

$$\text{Intake } (\mu g/kg/day) = \frac{UE\ (\mu g/g) \times CE\ (mg/kg/day)}{f \times 1000\ (mg/g)}$$
$$\times \frac{MW_d}{MW_m}$$

where UE is the urinary concentration of monoester per gram creatinine, CE is the creatinine excretion rate normalized by body weight, f is the ratio of urinary excretion to total elimination, and MW_d and MW_m are the molecular weights of the diesters and monoesters, respectively.

Table 2 shows the estimated ambient exposure to PAEs. As shown in Table 2, all estimated PAE intakes in the US population were lower than the tolerable daily intake (TDI) values settled by the EU Scientific Committee for Toxicity, Ecotoxicity and the Environment (BBP: 200 µg/kg/day, DBP: 100 µg/kg/day, DEHP: 37 µg/kg/day, DOP: 370 µg/kg/day, and DINP:150 µg/kg/day) (CSTEE, 1998), the reference dose (RfD) of the US EPA (BBP: 200 µg/kg/day, DBP: 100 µg/kg/day, and DEHP: 20 µg/kg/day) (US EPA, 2006) and the TDI values established by the Japanese Government (DEHP: 40–140 µg/kg/day and DINP: 150 µg/kg/day) (MHLW, 2002). Among these PAEs, DEHP is most commonly used plasticizer for flexible PVC formulations and is a widespread environmental contaminant (Kavlock et al., 2002c); however, the

estimated daily intake level of DEHP was not high as expected.

Koch et al. (2004a, 2003) and Barr et al. (2003) cast doubt on the sensitivity of the biomarker MEHP for assessing DEHP exposure, and they explored mono- (2-ethyl-5oxo-hexyl) phthalate (5oxo-MEHP) and mono- (2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) as additional biomarkers for DEHP. After a single oral dose of DEHP in a male volunteer, peak concentrations of MEHP, 5OH-MEHP, and 5oxo-MEHP were found in the serum after 2 h, and in urine after 2 h (MEHP) and 4 h (5OH-MEHP and 5oxo-MEHP). The major metabolite was MEHP in serum and 5OH-MEHP in urine (Koch et al., 2004a). Barr et al. (2003) analyzed 62 urine samples for metabolites of DEHP, and the mean urinary levels of 5oxo-MEHP and 5OH-MEHP were 4-fold higher than MEHP.

Koch et al. (2003) determined a median DEHP intake of 13.8 µg/kg/day based on urinary oxidative metabolites of DEHP, 5OH-MEHP and 5oxo-MEHP, in male and female Germans ($n = 85$; aged 18–40). Twelve percent of the subjects exceeded the TDI of the EU-CSTEE (37 µg/kg/day) and 31% of the subjects exceeded the RfD of the US EPA (20 µg/kg/day). For DBP, BBP, DEP, and DOP, the 95th percentile intake values were estimated to be 16.2, 2.5, 22.1, and 0.42 µg/kg/day, respectively. Subsequently, urine samples from 254 German children aged 3–14 were also analyzed for concentrations of these three metabolites of DEHP. The geometric means for MEHP, 5OH-MEHP and 5oxo-MEHP in urine were 7.9, 52.1, and 39.9 µg/L, respectively (Becker et al., 2004). The median daily intake of DEHP in children was estimated to be 7.7 µg/kg. Four children exceeded the TDI of the EU-CSTEE (37 µg/kg/day) and 26 children also exceeded the RfD of the US EPA (20 µg/kg/day) (Koch et al., 2006).

Although these findings showed that German populations could be exposed to DEHP at a higher level than previously estimated values (David et al., 2001; Kohn et al., 2000), these results should be interpreted carefully. In the above-mentioned equation, Kohn et al. (2000) and David et al. (2001) applied the fractional urinary excretion value ($f = 0.106$: MEHP) determined by Peck and Albro (1982). On the other hand, Koch et al. (2003) applied the fractional urinary excretion values ($f = 0.074$: 5OH-

Table 2

Comparison of calculated intakes of phthalates based on the geometric mean values for urinary metabolites and the tolerable daily intake levels as well as the reference dose of phthalates (in µg/kg/day)

PAEs	Estimated by David et al. (2001) for 289 US individuals (Blount et al., 2000)		Estimated by Kohn et al. (2000) for 2541 US individuals (Silva et al., 2004a)		TDI (EU) (CSTEE, 1998)	RfD (US) (US EPA, 2006)	TDI (Japan) (MHLW, 2002)
	Geometric mean	95th percentile	Geometric mean	95th percentile			
BBP	0.73	3.34	0.88	4.0	200	200	Not established
DBP	1.56	6.87	1.5	7.2	100	100	Not established
DEHP	0.60	3.05	0.71	3.6	37	20	40–140
DOP	<LOD	—	0.0096	0.96	370	Not established	Not established
DINP	0.21	1.08	<LOD	1.7	150	Not established	150

LOD, limit of detection.

MEHP, 0.055: 5oxo-MEHP and 0.024: MEHP) determined by Schmid and Schlatter (1985). Using different fractional urinary excretion values can yield several fold differences in estimated values even if the levels of the urinary metabolites are the same.

Table 3 shows a comparison of the estimated median exposure levels of DEHP. Koo and Lee (2005) and Fujimaki et al. (2006) applied the same fractional urinary excretion values of Koch et al. (2003) for calculating daily DEHP intake. Koo and Lee (2005) estimated daily intake of DEHP in Korean children aged 11–12 years old ($n = 150$) and in Korean women aged 20–73 years old ($n = 150$) with a fractional urinary excretion value of 0.024 for MEHP. Median intake levels of DEHP were estimated to be 6.0 μg/kg/day in children and 21.4 μg/kg/day in adult women. TDI of the EU (37 μg/kg/day) was reached at the 56th percentile for women and the 95th percentile for children. Fujimaki et al. (2006) estimated the daily intake of DEHP in forty pregnant Japanese women. The median concentrations of MEHP, 5OH-MEHP and 5oxo-MEHP in the urine were 9.83, 10.4, and 10.9 μg/L, respectively. The median DEHP intake based on MEHP, 5OH-MEHP, and 5oxo-MEHP were estimated to be 10.4 (3.45–41.6), 4.55 (0.66–17.9), and 3.51 (1.47–8.57) μg/kg/day, respectively. These two studies showed higher exposure levels than the previously estimated values in the US population (David et al., 2001; Kohn et al., 2000). Koo and Lee (2005) also showed that a different estimation model can yield 10-fold lower values when estimating DEHP intake, indicating that methods for estimation of daily intake values of PAEs remain inconsistent.

Recently, other secondary oxidized metabolites of DEHP have been recognized (Koch et al., 2005b). Although 5OH-MEHP and 5oxo-MEHP in the urine reflect short-term exposure levels of DEHP, other secondary oxidized metabolites of DEHP such as mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) and mono-[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) are considered excellent parameters for measurement of the time-weighted body burden of DEHP due to their long half-times of elimination. Biological monitoring in a German population ($n = 19$) indicated that 5cx-MEPP is the major urinary

metabolite of DEHP. Median concentrations of the metabolites of DEHP were 85.5 μg/L (5cx-MEPP), 47.5 μg/L (5OH-MEHP), 39.7 μg/L (5oxo-MEHP), 9.8 μg/L (MEHP) and 36.6 μg/L (2cx-MMHP) (Preuss et al., 2005). Furthermore, oxidized metabolites of DINP have been recently introduced as new biomarkers for measurement of DINP exposure (Koch and Angerer, 2007; Silva et al., 2006b). These new findings imply that more accurate methods for estimation of PAE exposure can be developed.

2.2. Exposure in fetuses and infants

PAE exposure to the fetus in utero is a great concern because some PAEs are considered to be developmental toxicants. Adibi et al. (2003) measured of urinary phthalate metabolites in pregnant women ($n = 26$) in New York. The median creatinine-adjusted concentrations of MEP, MBP, MBzP, and MEHP were 236, 42.6, 12.1, and 4.06 μg/g, respectively. Metabolites levels in pregnant women were comparable with those in US general population (Blount et al., 2000; Silva et al., 2004a). Another study in 24 mother–infant pairs confirmed DEHP and/or MEHP exposure during human pregnancies (Latini et al., 2003a). The mean DEHP concentrations in maternal plasma and cord plasma were 1.15 and 2.05 μg/mL, respectively, and the mean MEHP concentration was 0.68 μg/mL in both maternal plasma and cord plasma. The levels of phthalate metabolites in the amniotic fluid may reflect fetal exposure to PAEs. Only three metabolites, MEP, MBP, and/or MEHP, were detected in the amniotic fluid samples ($n = 54$). The levels of mono-methylphthalate (MMP), MBzP, McHP, MINP, MOP, 5OH-MEHP, and 5oxo-MEHP were under the limits of detection. Levels of MEP, MBP, and MEHP ranged from under the limits of detection to 9.0 ng/mL ($n = 13$), 263.9 ng/mL ($n = 50$), and 2.8 ng/mL ($n = 21$), respectively (Silva et al., 2004b). These studies suggest that human exposure to PAEs can begin in utero.

Breast milk and infant formula can be routes of PAE exposure for infants. Table 4 shows phthalate monoesters levels in human milk, infant formula, and consumer milk. Levels of phthalate monoesters in pooled breast milk

Table 3
Comparison of estimated mean daily intake of DEHP (μg/kg/day)

Metabolites	German[a] adults ($n = 85$) (Koch et al., 2003)	Korean[a] (Koo and Lee, 2005)		Japanese[a] pregnant women ($n = 40$) (Fujimaki et al., 2006)	US[b] adults ($n = 289$) (David et al., 2001)	US[b] aged ≥6 years ($n = 2541$) (Kohn et al., 2000)
		Adults (women) ($n = 150$)	Children ($n = 150$)			
MEHP	10.3 (38.3)	21.4 (158.4)	6.0 (37.2)	10.4	0.60 (3.05)	0.71 (3.6)
5OH-MEHP	13.5 (51.4)	No data	No data	4.55	No data	No data
5oxo-MEHP	14.2 (52.8)	No data	No data	3.51	No data	No data
Oxidative DEHP metabolites[c]	13.8 (52.1)	No data	No data	No data	No data	No data

Figures in parentheses show the 95th percentile.

[a] Applying the equation of David et al. (2001) and the fractional urinary excretion value determined by Schmid and Schlatter (1985).

[b] Applying the equation of David et al. (2001) and the fractional urinary excretion value determined by Peck and Albro (1982).

[c] Average of estimated intakes of DEHP based on 5OH-MEHP and 5oxo-MEHP.

Table 4
Phthalate monoester levels (μg/L) in human milk, infant formula and consumer milk

Monoester	Diester	Three pooled breast milk samples (Calafat et al., 2004b)	Thirty-six samples of Danish mother's milk (Mortensen et al., 2005)	Ten samples of infant formula (Mortensen et al., 2005)	Seven samples of consumer milk (Mortensen et al., 2005)
MMP	DMP	<LOD	0.17 ± 0.26[a]	<LOD	<LOD
MEP	DEP	<LOD	1.78 ± 2.74	<LOD	<LOD
MBP	DBP/ BBP	1.3 ± 1.5[a]	359 ± 1830	0.6–3.9[b]	1.4–2.8[b]
MBzP	BBP	<LOD	1.2 ± 1.6	<LOD	<LOD
MEHP	DEHP	7.8 ± 6.8	13 ± 11	5.6–9.1	7.1–9.9
MINP	DINP	15.9 ± 7.7	114 ± 69	<LOD	<LOD

LOD, limit of detection.
[a] Values are given as mean ± standard deviation.
[b] Values are given as range.

($n = 3$) were reported by Calafat et al. (2004b). A subsequent study for 36 individual human milk samples provided higher values for all metabolites; in particular, levels of MBP were two magnitudes higher (Mortensen et al., 2005) than that in the previous study by Calafat et al. (2004b). Phthalate metabolites in breast milk were detected in their free forms unlike the metabolites found in urine and blood. Therefore, infants may receive active PAE metabolites from breast milk on a daily basis. Only MBP and MEHP were detected in consumer milk and infant formula (Mortensen et al., 2005).

The levels of PAEs were determined for 27 infant formulae sold in several countries, and DEHP and DBP were found (Yano et al., 2005). The amounts of DEHP (34–281 ng/g) were much higher than DBP (15–77 ng/g). DEHP, DBP, and DEP were also found in a total of 86 human milk samples collected from 21 Canadian mothers over a 6-month postpartum period. DEHP was the major ester with a mean value of 222 ng/g (8–2920 ng/g), followed by DBP with a mean of 0.87 ng/g (undetectable to 11.39 ng/g). DEP with a mean of 0.31 ng/g (undetectable to 8.1 ng/g) was detected in only a small number of samples. Dimethyl phthalate (DMP), BBP, and DOP were not detected in any samples (Zhu et al., 2006). Table 5 presents estimated maximum daily intakes of PAEs in infants, which was calculated by assuming that the body weight of infants is 7 kg and the daily intake of milk is 700 mL. Although the total estimated maximum daily intake of DEHP in infants was generally less than in general adults (Koch et al., 2003), the estimated maximum daily intake per body weight was higher than adults due to the low body weight of the infants. Assuming that milk was the only exposure route for PAEs in the infants, it is likely that infants had less exposure to DBP and DEP than the general adult population (Koch et al., 2003). These studies suggest that some infants may also be exposed to DEHP at higher levels than the established safe standard levels.

2.3. Possible variation of PAE exposure

Some humans may be exposed to PAEs at higher level than the established safe standard levels. Measurements of urinary metabolites of PAEs have revealed notable differences in concentrations of specific metabolites based on age, gender and race (Blount et al., 2000; Silva et al., 2004a). Concentrations of MBP, MBzP, and MEHP were higher in the youngest age group (6–11 years) and decreased with age. Non-Hispanic blacks tended to have higher levels of phthalate metabolites than non-Hispanic whites or Mexican Americans. Females tended to have a higher level of phthalate metabolites than did males (Silva et al., 2004a). Blount et al. (2000) also indicated that women of reproductive age (20–40 years) had significantly higher levels of MBP than other age/gender groups. Measurement of the three urinary metabolites MEHP, 5OH-MEHP and 5oxo-MEHP in male and female children ($n = 254$) aged 3 to 14 showed that boys had higher concentrations of these three metabolites of DEHP than girls (Becker et al., 2004). The higher levels of PAE metabolites in the young age group may be due to a different food category, dairy products, or the use of PVC toys (CSTEE, 1998), and the higher levels of MBP in females may be

Table 5
Estimated maximum daily intake (μg/kg/day) of PAEs in infants and general German population

Compounds	Human milk ($n = 21$) (Zhu et al., 2006)	Infant formula ($n = 27$) (Yano et al., 2005)	General population ($n = 85$) (Koch et al., 2003)
DEHP	301 (41.1)	6.9	166 (52.1)
DBP	1.21 (0.12)	1.07	22.6 (16.2)
DEP	0.87	Not measured	69.3 (22.1)

Daily PAE intake levels were calculated by assuming that the average daily milk consumption is 700 mL (722 g: specific gravity of human milk = 1.031) and average body weight is 7 kg.
Figures in parentheses show 95th percentile.

due to use of cosmetic products that contain high levels of DBP (Koo and Lee, 2004).

Koo et al. (2002) approached this issue from a different point of view. Their statistical examination concluded that higher levels of MBP in urine were associated with a lower level of education (only a high school education) and/or lower family income (less than $1500) in the month before sampling. Slightly higher levels of MEP were found in urban populations, low income groups, and males. PAE exposure occurred from food, water, and indoor air, although dietary intake of PAEs from contaminated food was likely to be the largest source (Schettler, 2006). Education level and family income may therefore influence the dietary pattern.

It is still unknown whether the variations in these metabolites represent differences in the actual exposure levels. Metabolism of PAEs may vary by age, race, or sex; for example, the ratios of 5OH-MEHP/5oxo-MEHP and 5oxo-MEHP/MEHP decrease with increasing age (Becker et al., 2004). The mean relative ratios of urinary MEHP to 5OH-MEHP to 5oxo-MEHP were 1 to 7.1 to 4.9 in German male and female children and 1 to 3.4 to 2.1 in German male and female adults. This might indicate enhanced oxidative metabolism in children (Koch et al., 2004b). The ratios for urinary MEHP, 5OH-MEHP and 5oxo-MEHP in Japanese pregnant women were reported to be approximately 1 to 1 to 1 (Fujimaki et al., 2006). The variation seen in these three populations may be due to differences in the analytical methods; however, these variations in human populations are still not negligible for accurate risk assessment. Because the current estimates of PAE intake in humans can be imprecise and ADMEs of PAEs in each subpopulation are not clear, the significance of exposure to PAEs with regard to health effects is yet unknown.

2.4. Exposure from medical devices

DEHP has been used for a wide variety of PVC medical devices such as i.v. storage bags, blood storage bags, tubing sets, and neonatal intensive care units (NICUs), and known treatments that involve high DEHP exposures include blood exchange transfusions, extracorporeal membrane oxygenation and cardiovascular surgery.

Serum concentrations of DEHP were significantly increased in platelet donors and receptors (Buchta et al., 2005, 2003; Koch et al., 2005c). A median increase of 232% of serum DEHP was detected after plateletpheresis in healthy platelet donors (Buchta et al., 2003). Mean DEHP doses for discontinuous-flow platelet donors and continuous-flow platelet donors were 18.1 and 32.3 µg/kg/day on the day of apheresis, which were close to or exceeded health standard levels such as the TDI or RfD (Koch et al., 2005c).

Premature infants who experience medical procedures may have a higher risk of exposure to DEHP than the general population. Because the same size of each medical device is used for all ages, infants may receive a larger dose

of PAEs on a mg/kg basis than adults due to their smaller size. Calafat et al. (2004a) provided the first quantitative evidence confirming that infants who undergo intensive therapeutic medical interventions are exposed to higher concentrations of DEHP than the general population. They assessed exposure levels of DEHP in 6 premature newborns (23–26 weeks old) by measuring levels of urinary MEHP, 5OH-MEHP and 5oxo-MEHP. The geometric mean concentrations of MEHP (100 µg/L), 5oxo-MEHP (1617 µg/L), and 5OH-MEHP (2003 µg/L) were found to be one or two orders of magnitude higher than German children aged 3–5 (MEHP: 6.96 µg/L, 5OH-MEHP: 56.7 µg/L and 5oxo-MEHP:42.8 µg/L). Koch et al. (2005a) estimated DEHP exposure due to medical devices by using five major DEHP metabolites. Forty-five premature neonates (2–31 days old) with a gestational age of 25–40 weeks at birth were exposed to DEHP up to 100 times over the RfD value set by the US EPA depending on the intensity of medical care (median: 42 µg/kg/day; 95th percentile: 1780 µg/kg/day).

3. Health effects of PAEs in human populations

In the late 20th century, a few studies reported a relationship between environmental exposure of PAEs and human health. For example, Murature et al. (1987) reported that there was a negative correlation between DBP concentration in the cellular fraction of ejaculates and sperm production. Fredricsson et al. (1993) reported that human sperm motility was affected by DEHP and DBP. In females, decreased rates of pregnancy and higher levels of miscarriage in factory workers were associated with occupational exposure of DBP (Aldyreva et al., 1975). More recent studies in human males, females and infants are summarized below.

3.1. Studies of the male reproductive system

Table 6 shows a summary of studies of the male reproductive system in human populations. Two studies are available for 168 male subjects who were members of subfertile couples (Duty et al., 2003a,b). Eight urinary PAE metabolites, MEP, mono-methyl phthalate (MMP), MEHP, MBP, MBzP, MOP, MINP and McHP, were measured with a single spot urine sample. Urinary MEHP, MOP, MINP, or McHP showed no relevance to sperm parameters or DNA damage (Duty et al., 2003a,b). Urinary MBP was associated with lower sperm concentration and lower motility, and urinary MBzP was associated with lower sperm concentration. There was limited evidence suggesting an association of increased MMP with poor sperm morphology (Duty et al., 2003a). A neutral comet assay revealed that urinary MEP levels were associated with increased DNA damage in sperm (Duty et al., 2003b). This result was confirmed by a recent study in 379 men from an infertility clinic in which sperm DNA damage was associated with MEP (Hauser et al., 2007).

Table 6
Male reproductive effects in human populations

Compounds	Number of subjects	Related effects	Reference
Total PAEs[a]	$n = 21$	↓Sperm normal morphology, ↑Percent of single-stranded DNA in sperm	Rozati et al. (2002)
Phthalic acid	$n = 234$	↑Large testis[c], ↑Sperm motility[c]	Jonsson et al. (2005)
DEHP	$n = 37$	↓Semen volume, ↑Rate of sperm malformation	Zhang et al. (2006)
MEHP	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 74$	↓Plasma free testosterone	Pan et al. (2006)
%MEHP[b]	$n = 379$	↑Sperm DNA damage	Hauser et al. (2007)
MEP	$n = 168$	↑DNA damage in sperm	Duty et al. (2003b)
	$n = 234$	↑Large testis[c], ↓Sperm motility, ↓Luteinizing hormone	Jonsson et al. (2005)
	$n = 379$	↑DNA damage in sperm	Hauser et al. (2007)
	$n = 187$	↓Sperm linearity[d], ↑Straight-line velocity and curvilinear velocity of sperm[c,d]	Duty et al. (2004)
DBP	$n = 37$	↓Semen volume	Zhang et al. (2006)
MBP	$n = 168$	↓Sperm concentration, ↓Sperm motility	Duty et al. (2003a)
	$n = 463$	↓Sperm concentration, ↓Sperm motility	Hauser et al. (2006)
	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 74$	↓Plasma free testosterone	Pan et al. (2006)
	$n = 295$	↑Inhibin B level[c,d]	Duty et al. (2005)
MBzP	$n = 168$	↓Sperm concentration	Duty et al. (2003a)
	$n = 463$	↓Sperm concentration[d]	Hauser et al. (2006)
	$n = 187$	↓Straight-line velocity and curvilinear velocity of sperm[d]	Duty et al. (2004)
	$n = 295$	↓Follicle-stimulating hormone[c]	Duty et al. (2005)
MMP	$n = 168$	↑Poor sperm morphology[d]	Duty et al. (2003a)

[a] Total level of DMP, DEP, DBP, DEHP and DOP.

[b] The urinary concentrations of MEHP divided by sum of MEHP, 5OH-MEHP and 5oxo-MEHP concentrations and multiplied by 100.

[c] Data do not support the association of PAEs with reproductive adverse effects in male human populations.

[d] Only suggestive association was observed (statistically not significant).

In another study, semen volume, sperm concentration, motility, sperm chromatin integrity and biochemical markers of epididymal and prostatic function were analyzed together with MEP, MEHP, MBzP, MBP, and phthalic acid levels in urine in 234 young Swedish men (Jonsson et al., 2005). Urinary MEP level was associated with fewer motile sperm, more immotile sperm, and lower serum luteinizing hormone (LH) values. However, higher phthalic acid levels were associated with more motile sperm and fewer immotile sperm; therefore, the results for phthalic acid were opposite what had been expected.

A similar study was conducted in 463 male partners of subfertile couples (Hauser et al., 2006). Phthalate metabolites were measured in a single spot urine sample. There were dose–response relationships of MBP with low sperm concentration and motility. There was suggestive evidence of an association between the highest MBzP quartile and low sperm concentration. There were no relationships between MEP, MMP, MEHP or oxidative DEHP metabolites with any of the semen parameters.

Although there were associations between some metabolites of PAEs and sperm count, motility, or morphology, no statistically significant associations between MEP, MBzP, MBP, MEHP, or MMP and sperm progression, sperm vigor, or swimming pattern were observed in 187 subjects. There were only suggestive associations as follows: negative associations between MBzP with straight-line velocity (VSL) or curvilinear velocity (VCL), between MBP with VSL and VCL and between MEHP with VSL and VCL. MEP was positively associated with VSL and VCL but negatively associated with linearity (Duty et al., 2004).

Duty et al. (2005) explored the relationship between urinary phthalate monoester concentrations and serum levels of reproductive hormones in 295 men. In their previous studies (Duty et al., 2003a,b), MBP and MBzP were associated with sperm parameters, and the investigators had hypothesized that inhibin B, a sensitive marker of impaired spermatogenesis (Uhler et al., 2003), would be inversely associated with MBP and MBzP. However, MBP exposure was associated with increased inhibin B, although this was of borderline significance. Additionally, MBzP exposure was significantly associated with a decrease in serum follicle-stimulating hormone (FSH) level. The serum FSH level has been used as a marker of spermatogenesis for infertile males in clinical evaluation (Subhan et al., 1995), and it is increased in comparison to normal males (Sina et al., 1975). Therefore, the hormone concentrations did not change in the expected patterns.

DEHP is known to cause adverse effects on the male reproductive system in rodents (Gray et al., 2000), and DNA damage in human lymphocytes is also induced by DEHP and MEHP (Anderson et al., 1999). A Hershberger assay with DEHP or MEHP showed anti-androgenic effects in castrated rats (Stroheker et al., 2005; Lee and Koo, 2007). However, only a few studies have suggested that DEHP could be a reproductive toxicant in humans. Urine and blood samples from 74 male workers at a factory producing unfoamed polyvinyl chloride flooring exposed to DBP and DEHP were compared with samples from 63 unexposed male workers. The exposed workers had significantly elevated concentrations of MBP (644.3 vs. 129.6 µg/g creatinine) and MEHP (565.7 vs. 5.7 µg/g

creatinine) in their urine. The plasma free testosterone level was significantly lower (8.4 vs. 9.7 μg/g creatinine) in the exposed workers than in the unexposed workers. Free testosterone was negatively correlated to MBP and MEHP in the exposed worker group (Pan et al., 2006). Another recent study showed that although the urinary MEHP concentration was not associated with sperm DNA damage, the percentage of DEHP metabolites excreted as MEHP (MEHP%) was associated with increased sperm DNA damage. It is of interest that the oxidative metabolites had inverse relationships with sperm DNA damage (Hauser et al., 2007).

Unlike other studies, the following two studies used diester concentrations for measurement of PAEs. Rozati et al. (2002) reported that the concentration of total PAEs (DMP, DEP, DBP, BBP, DEHP, and DOP) in the seminal plasma was significantly higher in infertile men ($n = 21$) compared to controls ($n = 32$). Correlations were observed between seminal PAEs and sperm normal morphology ($r = -0.769$, $p < .001$), in addition to the % of single-stranded DNA in the sperm ($r = 0.855$, $p < .001$). This study examined only total PAEs, and relationships between individual PAEs and sperm parameters were not identified. Another study in a human male population was carried out by measurement of semen parameters and DEHP, DBP, and DEP in human semen ($n = 37$) (Zhang et al., 2006). The three PAEs were detected in most of the samples, and mean levels of DEHP, DBP, and DEP were 0.28, 0.16, and 0.47 μg/L, respectively. There was a negative correlation between semen volume and concentration of DBP or DEHP. There was also a positive association between the rate of sperm malformation and DEHP concentrations. These diester concentrations may directly reflect PAE exposure levels.

Animal data have suggested that mature exposure to DBP and DEHP affects sperm parameters (Agarwal et al., 1986; Higuchi et al., 2003). Dietary exposure of mature male F344 rats (15–16 weeks old) to DEHP (0–20,000 ppm) for 60 consecutive days resulted in a dose dependent reduction in testis, epididymis and prostate weights at 5000 and 20,000 ppm (284.1 and 1156.4 mg/kg/day). Epididymal sperm density and motility were also reduced and there was an increased occurrence of abnormal sperm at 20,000 ppm (Agarwal et al., 1986). Exposure of BBP from adolescence to adulthood showed changes in reproductive hormones in CD(SD)IGS rats at 100 and 500 mg/kg/day (Nagao et al., 2000). In Dutch-Belted rabbits, exposure of DBP during adolescence and in adulthood decreased the amount of normal sperm whereas in utero exposure of DBP decreased the amount of normal sperm, sperm counts, ejaculated volume, and accessory gland weight (Higuchi et al., 2003). Preadolescent male rats appear to have a greater sensitivity to the adverse testicular effects of DEHP than older rats. Akingbemi et al. (2001) demonstrated that preadolescent male rats (21 days old) were more sensitive than young adult animals (62 days old) to 14- or 28-day DEHP exposures that

induced decreases in Leydig cell production of testosterone. PAE effects on male reproductive organs could be influenced by the stage of development, but the data also support the possibility that mature animals are susceptible to PAE exposure. The studies in human populations were in accord with these animal data.

Some studies in human populations have suggested associations between MEP, a metabolite of DEP, and changes in sperm; however, these results regarding to MEP are not supported by animal studies. According to Foster et al. (1980), oral dosing of DEP (1600 mg/kg/day) for 4 days did not damage the testes in young SD rats. In another study, male and female CD-1 mice were given diets with DEP (0–2.5%) for 7 days prior to and during a 98-day cohabitation period. There were no apparent effects on reproductive function in animals exposed to DEP (Lamb et al., 1987).

Furthermore, studies in rodents may have little relevance to humans for the reason that DEHP and DINP do not cause reproductive effects in non-human primates. Pugh et al. (2000) showed no evidence of testicular lesions in young adult cynomolgus monkeys (~2 years old) gavage dosed with 500 mg/kg bw/day DEHP and DINP for 14 days. A study with matured marmosets (12–15 months old) showed that repeated dosing of DEHP at up to 2500 mg/kg bw/day for 13 weeks resulted in no differences in testicular weight, prostate weight, blood testosterone levels, blood estradiol levels or any other aspect of the reproductive system (Kurata et al., 1998). DEHP treatment up to 2500 mg/kg bw/day in marmosets from weaning (3 months old) to sexual maturation (18 months old) produced no evidence of testicular damage. Sperm head counts, zinc levels, glutathione levels and testicular enzyme activities were also not affected (Tomonari et al., 2006). In contrast to data from rabbits and rodents, no testicular effects of DEHP or DINP were found in non-human primates at any ages. The current understanding of how PAEs affects semen parameters, sperm DNA damage, and hormones in human populations is limited and further investigation is required.

3.2. Studies of the female reproductive system

Studies of adult female humans are less numerous than those of adult males. Cobellis et al. (2003) compared plasma concentrations of DEHP and MEHP in endometriotic women ($n = 55$) with control women ($n = 24$), and higher plasma DEHP concentrations were observed in endometriotic women. Similar results were observed in a recent study reported by Reddy et al. (2006). The investigators collected blood samples from 49 infertile women with endometriosis (the study group), 38 infertile women without endometriosis (control group I) and 21 women with proven fertility (control group II). Women with endometriosis showed significantly higher concentrations of DBP, BBP, DOP, and DEHP when compared to both control groups. Upon analysis of cord blood samples of 84 newborns, Latini et al. (2003b) revealed that MEHP-positive

infants had a lower gestational age (38.16 ± 2.34 weeks) than MEHP-negative infants (39.35 ± 1.35 weeks). Intra-uterine inflammation due to DEHP and/or MEHP exposure may be a risk factor for prematurity because intrauterine infection/inflammation is a major cause of premature labor. These studies suggest that DEHP may play a role in inducing the intrauterine inflammatory process.

Thelarche, premature breast development, is the growth of mammary tissue in young girls without other manifestations of puberty. Colon et al. (2000) analyzed serum samples from 41 Puerto Rican thelarche patients and 35 age matched controls. Significantly higher levels of DMP, DEP, DBP, DEHP, and MEHP were found in 28 (68%) samples from thelarche patients. This study suggested a possible association between PAEs and premature breast development. However, McKee et al. (2004) stated that the association between PAE exposure and thelarche seems highly unlikely for two reasons. First, the reported exposure levels of PAEs may have reflected contamination since they were very high when compared to recent exposure information. Second, toxicological evidence from the laboratory studies described below do not support any influence on female sexual development.

DEHP exposure at 2000 mg/kg/day for 1–12 days in mature SD rats resulted in decreased serum estradiol levels, prolonged estrous cycles and no ovulation (Davis et al., 1994). A two generation reproductive study in SD rats revealed that oral doses of 500 mg/kg/day BBP caused atrophy of the ovary in one female and significant decreases in absolute and relative ovary weights. However oral doses of up to 500 mg/kg/day BBP did not affect estrous cycles in SD rats (Nagao et al., 2000). Similarly, when DEHP was administered to rats over two generations at up to 9000 ppm (about 900 mg/kg/day) in the diet, there were no effects on the pattern and duration of the estrous cycle in F0 female rats (Schilling et al., 1999). Histological changes in female reproductive organs were also not observed after exposure to di-*n*-propyl phthalate, DBP, di-*n*-pentyl phthalate, DHP, or DEHP (Heindel et al., 1989; Lamb et al., 1987). Although some PAEs have been reported to be weakly estrogenic in estrogen-responsive

human breast cancer cells (Jobling et al., 1995; Sonnensch-ein et al., 1995; Soto et al., 1995; Zacharewski et al., 1998) and/or in a recombinant yeast screen (Coldham et al., 1997; Harris et al., 1997), no PAEs showed any estrogenic response upon in vivo uterotrophic or vaginal cornification assay (Zacharewski et al., 1998). Thus, there is no evidence that PAEs influence the timing of female sexual development in laboratory studies.

3.3. Studies in human infants

Anogenital distance (AGD) is a developmental landmark for the differentiation of the external genitalia and is commonly used as a hormonally sensitive parameter of sex differentiation in rodents. AGD in male rats is normally about twice that in females, and a similar sex difference is observed in humans (Salazar-Martinez et al., 2004). Many studies in male rodents reported a reduction of AGD after prenatal exposure to PAEs (Table 7). Chemicals that adversely affect human sex differentiation (Schardein, 2000) also produce predictable alterations of this process in rodents (Gray et al., 1994). In a Hershberger assay, significant decreases in seminal vesicles, ventral prostate, levator ani/bulbocavernosus muscles weights were observed in animals treated with DEHP, DBP, DINP, di-isodecyl phthalate or MEHP, which suggest that some phthalates possess anti-androgenic activity (Lee and Koo, 2007). Swan et al. (2005) presented the first study of AGD and other genital measurements in relation to PAE exposure in a human population. AGD data were obtained for 134 boys of 2–36 months of age. Mother's urine during pregnancy was assayed for phthalate metabolites. Urinary concentrations of four phthalate metabolites, MEP, MBP, MBzP, and mono-isobutyl phthalate (MiBP), were negatively related to the anogenital index (AGI) which is a weight-normalized index of AGD [AGD/weight (mm/kg)].

In rats, undescended testes were observed in male pups after maternal dosing of BBP, MBzP, DEHP, DBP, or MBP (Table 7). Main et al. (2006) investigated whether phthalate monoesters in human breast milk had any relation to cryptorchidism in newborn boys (1–3 months of

Table 7
Decreased AGD and undescended testes observed in experimental animals

Compounds	Animals	Days of administration	Route	Dose (mg/kg/day)	Decreased male AGD	Undescended testes	Reference
BBP	Wistar rat	GDs 15–17	Gavage	500	+	+	Ema and Miyawaki (2002)
				1000	+	+	
MBzP	Wistar rat	GDs 15–17	Gavage	250	+	+	Ema et al. (2003)
				375	+	+	
DEHP	SD rat	GD 2-PND 21	Gavage	750	+		Moore et al. (2001)
				1500	+	+	
DBP	Wistar rat	GDs 11–21	Diet	555	+	+	Ema et al. (1998)
				661	+	+	
DBP	Wistar rat	GDs 15–17	Gavage	500	+	+	Ema et al. (2000)
				1500	+	+	
MBP	Wistar rat	GDs 15–17	Gavage	250	+	+	Ema and Miyawaki (2001)
				500	+	+	

age). The median levels of MMP, MEP, MBP, MBzP, MEHP, and MINP in breast milk were 0.10, 0.95, 9.6, 1.2, 11, and 95 μg/L, respectively. No association was found between phthalate monoester levels and cryptorchidism. However, there were positive correlations for MEP and MBP with sex hormone-binding globulin, MMP, MEP, and MBP with the ratio of LH/free testosterone, and MINP with LH. MBP was negatively correlated with free testosterone. These mother–son cohort studies provided evidence that testicular and genital development may also be vulnerable to perinatal exposure to PAEs.

Although these two studies of human infants indicate possible associations between PAE exposure and the development of the human reproductive system, two follow-up studies of adolescents exposed to DEHP from medical devices as neonates showed no significant adverse effects on their maturity or sexual activity. A comparison of very low birth weight infants who had undergone neonatal intensive care and infants with normal birth weights showed that there were no differences in the rates of sexual intercourse, pregnancy, or live births when the infants became young adults (Hack et al., 2002). Another study indicated that adolescents exposed to DEHP as neonates showed no significant adverse effects on physical growth and pubertal maturity. Thirteen male and 6 female subjects of 14–16 years of age who had undergone extracorporeal membrane oxygenation as neonates had a complete physical examination to evaluate the long-term toxicity of DEHP in infants. Thyroid, liver, renal, and male and female gonadal functions tested were within normal ranges for the given age and sex distribution (Rais-Bahrami et al., 2004).

4. Overall conclusions

In conclusion, exposure data in human populations indicate that the current methodology of estimation of PAEs is inconsistent. It is important to obtain improved data on human PAE exposure and a better understanding of the toxicokinetics of PAEs in each subpopulation. Oxidized metabolites of DEHP and DINP were recently recognized as the major urinary metabolites in humans (Barr et al., 2003; Koch and Angerer, 2007; Koch et al., 2004a, 2005b). These findings could be useful to establish new hypotheses for laboratory studies. Hauser et al. (2007) found that oxidative metabolites of DEHP had a negative association with sperm DNA damage, suggesting that the oxidation of MEHP to 5OH-MEHP and 5oxo-MEHP is protective against sperm DNA damage. However, in an in vitro study, 5OH-MEHP and 5oxo-MEHP, but not DEHP or MEHP, were anti-androgenic (Stroheker et al., 2005). The relevance of this in vitro study to findings in human populations is not clear. Therefore, further studies are required to facilitate accurate risk assessments for human health.

Studies of health effects of PAEs in humans have remained controversial due to limitations of the study designs. Some findings in human populations are consis-

tent with animal data suggesting that PAEs and their metabolites produce toxic effects in the reproductive system. However, it is not yet possible to conclude whether phthalate exposure is harmful for human reproduction. Studies in humans have to be interpreted cautiously because they are conducted in a limited number of subjects. Spot samples only reflect recent phthalate exposure due to the short half-life and it has not yet been confirmed whether point estimates are representative of patterns of long exposure, although reproducibility was found for urinary phthalate monoester levels over two consecutive days (Hoppin et al., 2002). The timing of exposure is a critical factor for decreased AGD in animal studies (Ema and Miyawaki, 2001); however, the stage of fetal development was unknown at the time of urine sampling in the study of Swan et al. (2005). Further studies need to be conducted to confirm these results in human populations and identify the potential mechanisms of interaction.

The studies in human populations reviewed in this paper are useful for showing the strength of associations. Evidence from human studies is preferred for risk assessment as long as it is obtained humanely. It is sometimes claimed that the use of animal data for estimating human risk dose not provide strong scientific support. However, because it is difficult to find alternative methods to test the direct toxic effects of chemicals, continuance of studies in animals is required for risk assessment of chemicals including PAEs.

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